



# Bacterial, but not baculoviral infections stimulate *Hemolin* expression in noctuid moths

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## ABSTRACT

Lepidopteran larvae are regularly infected by baculoviruses during feeding on infected plants. The differences in sensitivity to these infections can be substantial, even among closely related species. For example, the noctuids Cotton bollworm (*Helicoverpa zea*) and Tobacco budworm (*Heliothis virescens*), have a 1000-fold difference in sensitivity to *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) infection.

Recent data were interpreted to indicate that the lepidopteran immunoglobulin protein, *Hemolin*, is synthesized upon viral injection and therefore to participate in anti-viral responses. To investigate whether *Hemolin* synthesis is affected by a natural viral infection, specific transcription in fat bodies and hemocytes of *H. zea* and *H. virescens* larvae was monitored following *per os* infection with the baculovirus HZSNPV (*H. zea* single nucleopolyhedrovirus). Both moths showed the same expression pattern as seen in uninfected animals and coincided with ecdysone responses, previously known to induce *Hemolin* expression. In contrast, injection of lyophilized *Micrococcus luteus* resulted in increased *Hemolin* expression supporting a role for *Hemolin* as an immuno-responsive protein in these species.

The combined data are consistent with the suggestion that while *Hemolin* seems to participate in the response to virus infection in the superfamily Bombycoidea, this is not true in the Noctuoidea.

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## 1. Introduction

Insect immune responses involved in virus defense have lately received increased focus. While the pathways and effector molecules active in defense against bacteria and fungi are well elaborated [1,2], we are only in the beginning of understanding how the innate immune system protects insects from viral infection [3]. Knowledge about the insect immune responses to bacteria and fungi has led to important advances in vertebrate innate immunity; therefore, we expect research on insect viral immunity to have the same effect.

The baculoviruses (Baculoviridae) are a family of viruses that infect arthropods, mainly insects and in particular Lepidoptera [4]. Although the use of baculoviruses in pest control has led to significant knowledge of viral pathobiology and ecology in Lepidoptera [4], little is known about the host immune responses towards viral infections. The anti-viral mechanism of host-cell transcription down-regulation, leading to global translational

arrest and apoptosis, is well studied in mammals [5]. Likewise; in Lepidoptera, one of the major responses against virus infection is the apoptosis of infected cells [6]. This is apparent in experiments where baculovirus-encoded anti-apoptotic genes have been deleted genetically or their products silenced by RNA interference (RNAi). For example, the yields of baculovirus decreased 100-fold and the lethal dose required for killing larvae increased more than 1000-fold after mutation of the anti-apoptotic gene p35 in *Spodoptera frugiperda* [7]. However, in addition to apoptosis, hitherto unknown factors also playing a role in the response have been suggested [6,8].

The lepidopteran-specific immunoglobulin protein *Hemolin* was found as the most abundant bacteria-induced protein in *Hyalophora cecropia* and *Manduca sexta* [9–11]. Bacteria-induced *Hemolin* expression has since been documented in several moth species and recently *Hemolin* involvement in anti-viral defense was suggested [12]. Baculovirus infection up-regulated the expression of *Hemolin* in the Chinese oak silkworm *Antheraea pernyi* and was confirmed by RNAi of *Hemolin* that resulted in accelerated death of virus-infected *A. pernyi* pupae [13]. *Hemolin* involvement in viral infection also was seen for polydnavirus, a virus attached to the injected eggs of parasitic wasps to interfere with the host immune responses. A polydnavirus protein from the

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parasitic wasp *Cotesia congregata* binds to Hemolin from *M. sexta* and inhibits the normal Hemolin functions of binding LPS and agglutination of bacteria [14].

A robust antibacterial response has been documented in *Heliothis virescens* [15,16], but far less is known about the larval response to baculoviral infection; although evidence exists that dietary selenium may be required to resist infection [17,18] and that the plasma enzyme prophenoloxidase may possess virucidal activity against baculoviruses [19]. Thus, *per os* HzSNPV infection of 5th instar *H. virescens* larvae did not alter expression of prophenoloxidase subunits in fat bodies or in hemocytes; however the expression of prophenoloxidase-1 was mildly suppressed when 4th instar larvae were infected though the expression of prophenoloxidase-2 was unchanged [18]. In contrast, bacterial elicitation of 5th instar *H. virescens* larvae significantly elevated hemocyte and fat body transcript levels of prophenoloxidase-1, but not of prophenoloxidase-2 [18]. While prophenoloxidase subunits are expressed constitutively at high levels in larval *H. virescens* hemocytes and fat bodies, the prophenoloxidase-1 subunit was thus differentially regulated by bacterial and baculoviral infection.

The *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) is the most extensively studied virus and infects primarily noctuids [20,21]. In a comparative study of AcMNPV-pathogenicity, baculovirus-infected cells were encapsulated by hemocytes and cleared from the hemocoel in *Helicoverpa zea*, but not in *H. virescens*, resulting in a 1000-fold increased resistance to mortal infection for the former [22].

To determine whether Hemolin plays a role in anti-viral immunity of noctuid moths and perhaps be a key factor in the differences in resistance observed, we cloned the Hemolin cDNAs from *H. zea* and *H. virescens*, and analyzed their expression in response to baculoviral and bacterial infection.

## 2. Material and methods

### 2.1. Insects, infections and tissue sample collections

*H. virescens* and *H. zea* eggs were received from the North Carolina State Univ. Dept. of Entomology Insectary (Raleigh, NC) and reared individually on an artificial wheat-germ-based diet (BioServe, Frenchtown, NJ) under standard conditions of 14 h:10 h (L:D) photoperiod, 55% RH, 28 °C [23]. For baculoviral response, newly moulted larvae were infected *per os* with an LC<sub>95</sub> (concentration killing 95% of larvae) at either  $5 \times 10^8$  (4th instar) or  $1 \times 10^9$  (5th instar) polyhedra/ml of HzSNPV or mock-infected according to Popham et al. [23]. Larvae were infected with virus mixed with food coloring visible through the integument. Additional trays of larvae were infected to confirm that the virus killed at the expected rate. By 60 h post-infection at the latest, larvae were showing clear signs of infection such as moribund behavior and an absence of burrowing. To activate the antibacterial immune response, newly moulted 5th instar larvae were punctured with a tungsten needle dipped into a suspension of PBS and heat-killed *Escherichia coli* and *Micrococcus luteus* [24]. *Micrococcus luteus* was purchased from Sigma Chemicals as *Micrococcus lysodeikticus* ATCC No. 4698, but is a synonym of *Micrococcus luteus*. Mock-infected controls received a sterile puncture. Tissues were dissected in cold PBS, put in RNeasy lysis buffer (Qiagen) and frozen at -84 °C for later isolation of RNA using RNeasy spin columns (Qiagen).

### 2.2. Cloning of Hemolin

*H. virescens* DNA (45 ng) was used to clone a fragment corresponding to exon 4 of Hemolin by using PCR supermix (Invitrogen). Forward primer Hem Ex4 HvF: 5'-GGT ACA AAA ATG

GCC AAC C-3' (nucleotide positions 227–245 in Fig. 1) and reverse primer Hem Ex4 HvR: 5'-GCT TTA GCC ATC ATA TCG TTG C-3' (nt pos 761–782) were designed based on the partial Hemolin cDNA sequence EF537866 from *H. virescens* (M. Geber, I. Faye, O. Terenius, direct submission). Thermal cycle was 94 °C for 30 s, followed by 30 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min, and final extension of 72 °C for 25 min. PCR products were cloned into a TOPO-T/A cloning vector (Invitrogen) and several independent clones were subjected to sequencing at Laguna Scientific, CA, USA.

To obtain full-length cDNA, 5'- and 3'-RACE were conducted with a SMART cDNA RACE kit (Clontech) using nested PCR. The first primer set was 5'RACE 1: 5'-CTA TGT TAC CAG CCT TCT CCC A-3' (nt pos 254–274) and 3'RACE 1: 5'-GTC AGG TGA AGA TGT GGT GTT GG-3' (nt pos 667–688) and the second primer set was 5'RACE 2: 5'-GGT TGG CCA TTT TTG TAC C-3' (nt pos 227–245) and 3'RACE 2: 5'-GCA ACG ATA TGA TGG CTA AAG C-3' (nt pos 761–782). PCR products were cloned into a TOPO-T/A cloning vector and several independent clones were subjected to sequence analysis. Using the sequence data obtained by RACE, new primers were designed outside the coding region and the full-length coding region of Hemolin was cloned and re-sequenced. The primers used to clone the entire *H. virescens* Hemolin sequence was also used to amplify Hemolin from *H. zea*.

### 2.3. qRT-PCR

#### 2.3.1. RNA isolation and reverse transcription

Total RNA was extracted from fat bodies and hemocytes using the RNeasy<sup>TM</sup> kit (Qiagen). RNA was reverse transcribed using the Qiagen Omniscript<sup>®</sup> Reverse Transcription kit. The reactions were carried out in a total volume of 20 µl using the cDNA synthase buffer, dNTP mix, oligo-dT, RT enhancer, Verso Enzyme mix and 5 µl of template RNA. The reaction was incubated at 37 °C for 60 min and inactivated at 95 °C for 5 min. The samples were either used immediately for qPCR or frozen at -20 °C until use. The expression of Hemolin transcripts relative to a house-keeping ribosomal protein transcript was determined using quantitative real-time PCR (qPCR). Reactions were performed in the Eppendorf Master Cycle Rep Realplex 4S and analyzed with the Realplex software. The qPCR reactions were performed using the Qiagen QuantiFast<sup>TM</sup> SYBR Green<sup>®</sup> qRT-PCR Kit and the Eppendorf twin.tec<sup>®</sup> 96 well PCR plates. The sequences of the primers used in the reactions were for *H. virescens* Hemolin transcript measurements: *Hv Hemolin* forward (TGG AGT CGG CAA GAA ACA GAC TCA), *Hv Hemolin* reverse (AGG CCA GTA ACT TGG CAA GGG ATA), *HvRPL4* forward (AGA TGC TGA ACG TGG ACA AGC TGA) and *HvRPL4* reverse (TTC AGC GGG TTG AGT TTC CTG GTA). The same primers were used for the determination of *H. zea* Hemolin transcript level, except that a different internal standard was used, *HvRPL21* forward (CGT CCG CAT TGA ACA CAT CAA GCA) and *HvRPL21* reverse (TGG GAG CCA ATA GCA CTG GTT TCT). The specificity of each primer pair was confirmed using agarose gel electrophoresis and by performing melting curve analysis on the completed qPCR reactions. Negative controls consisting of no-template reactions were performed for each primer pair. The PCR reaction was performed at 95 °C for 15 min followed by 50 cycles of 95 °C for 15 s, 55 °C for 30 s, and 72 °C for 30 s. All reactions were performed in duplicate and the *HvRPL4* normalized expression ratio was calculated using the  $\Delta\Delta C_T$  method [25]. Infection time-courses and dissections were repeated three separate times.

#### 2.3.2. Statistical comparisons

Triplicate real-time-PCR data were analyzed as a randomized complete block design ANOVA. All data was log transformed and

For amino acid sequence prediction of N-glycosylation and cAMP/cGMP sites, Proscan ([http://npsapbil.ibcp.fr/cgibin/npsa\\_automat.pl?page=npsa\\_prosite.html](http://npsapbil.ibcp.fr/cgibin/npsa_automat.pl?page=npsa_prosite.html)) [26] was used. For amino acid sequence prediction of signal sequence, SignalP (<http://www.cbs.dtu.dk/services/SignalP/>) [27] was used.

The identity and similarity of the nucleotide and amino acid sequences were analyzed using Needle [28]. The following settings were used: for nucleotides, matrix EDNAFULL, gap opening penalty 10.00, and gap extension penalty 0.5; for amino acids, matrix EBL0SUM62, gap opening penalty 10.00, and gap extension penalty 0.5.

Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4 [29]. Amino acid sequences were aligned by ClustalW 1.83 [30] using protein weight matrix Gonnet, with a gap opening penalty 10.00 and a gap extension penalty 0.2.

**Fig. 1.** cDNA and deduced amino acid sequences of *Hemolin* from *Helicoverpa zea* and *Heliothis virescens*. The amino acid signal sequences and cysteine residues forming disulfide bridges are underlined. Amino acids differing between the two species are shown in bold with yellow background and those with non-preferred substitutions are in addition written in italics. The polyadenylation signals (ATTTAA) are indicated by double underline.

Hv	209	-	A	Q	T	P	A	S	G	E	D	V	V	L	A	E	H	F	L	K	<b>S</b>	M	-	228																			
Hv	659	-	G	C	C	A	A	A	C	C	C	T	G	C	T	T	C	T	G	T	G	A	A	G	A	G	T	A	T	G	-	718											
Hv	652	-	G	C	C	A	A	A	C	C	C	T	G	C	A	G	T	G	T	G	T	G	T	G	G	C	A	A	T	T	C	T	G	A	A	G	G	C	A	T	G	-	711
Hv	206	-	A	Q	T	P	A	S	G	E	D	V	V	L	A	E	H	F	L	K	<b>A</b>	M	-	225																			
3' RACE 1st																																											
Hv	229	-	V	Q	D	K	T	P	Q	N	G	E	L	V	P	Q	Y	V	S	N	D	M	-	248																			
Hv	719	-	G	T	G	C	A	G	G	A	T	C	C	G	A	A	T	G	G	G	A	G	T	T	A	G	T	G	T	T	A	G	T	G	T	G	T	G	T	G	-	778	
Hv	712	-	G	T	C	C	A	G	A	C	T	C	T	C	A	A	T	G	G	G	A	G	T	G	T	G	T	G	T	G	T	G	T	G	T	G	T	G	-	771			
Hv	226	-	V	Q	D	K	T	P	Q	N	G	E	L	V	P	Q	Y	V	S	N	D	M	-	245																			
Ex4 R and 3' RACE 2nd																																											
Hv	249	-	M	A	K	A	G	D	V	T	M	I	Y	C	I	Y	G	G	T	P	L	G	-	268																			
Hv	779	-	A	T	G	G	C	A	A	A	G	C	T	G	T	G	A	T	G	T	T	A	C	C	A	T	G	T	T	A	C	C	A	T	G	T	G	-	838				
Hv	772	-	A	T	G	G	C	A	A	A	G	C	T	G	T	G	A	T	G	T	T	A	C	C	A	T	G	T	T	A	C	C	A	T	G	T	G	-	831				
Hv	246	-	M	A	K	A	G	D	V	T	M	I	Y	C	I	Y	G	G	T	P	L	G	-	265																			
Hv	269	-	F	P	S	W	Y	K	D	G	K	D	A	D	G	K	P	G	D	R	V	T	-	288																			
Hv	839	-	T	T	C	C	A	A	G	T	G	T	C	A	A	A	G	A	C	G	G	C	A	A	G	C	C	T	G	G	A	C	C	A	G	T	C	A	-	898			
Hv	832	-	T	T	C	C	A	A	G	T	G	T	C	A	A	A	G	A	C	G	G	C	A	A	G	C	C	T	G	G	A	C	C	A	G	T	C	A	-	891			
Hv	266	-	F	P	S	W	Y	K	D	G	K	D	A	D	G	K	P	G	D	R	V	T	-	285																			
Hv	289	-	A	Y	N	R	T	S	G	K	R	L	L	I	K	E	T	L	L	E	D	A	-	308																			
Hv	899	-	G	C	A	T	A	C	A	A	G	G	A	C	A	G	G	C	T	G	C	A	A	G	A	C	C	T	T	C	T	G	A	A	G	A	C	G	-	958			
Hv	892	-	G	C	A	T	A	C	A	A	G	G	A	C	A	G	A	C	C	T	G	C	A	A	G	A	C	C	T	T	C	T	G	A	A	G	A	C	-	951			
Hv	286	-	A	Y	N	R	T	S	G	K	R	L	L	I	K	E	T	L	L	E	D	A	-	305																			
Hv	309	-	G	E	Y	<b>A</b>	<b>C</b>	<b>I</b>	V	D	N	G	V	G	K	K	Q	T	H	<b>K</b>	M	R	-	328																			
Hv	959	-	G	G	T	G	A	T	A	C	G	C	T	G	C	A	A	T	G	G	C	G	T	C	G	G	A	A	A	A	C	A	A	A	C	A	A	A	-	1018			
Hv	952	-	G	G	T	G	A	T	A	C	G	C	T	G	C	A	A	T	G	G	C	G	T	C	G	G	A	A	A	A	C	A	A	A	C	A	A	-	1011				
Hv	306	-	G	E	Y	<b>K</b>	<b>C</b>	<b>V</b>	V	D	N	G	V	G	K	K	Q	T	H	<b>T</b>	M	R	-	325																			
qRT-PCR Hv Hem F																																											
Hv	329	-	L	T	V	V	S	A	P	K	L	S	K	K	<b>A</b>	E	K	V	<b>V</b>	<b>T</b>	<b>V</b>	K	-	348																			
Hv	1019	-	T	T	G	A	C	T	G	T	A	C	G	T	G	C	A	A	A	A	A	A	C	T	C	T	C	A	A	A	A	A	A	A	A	A	-	1078					
Hv	1012	-	C	T	G	A	C	T	G	T	T	G	T	A	C	G	T	G	C	C	C	A	A	A	C	T	C	T	C	A	A	A	A	A	A	A	-	1071					
Hv	326	-	L	T	V	V	S	A	P	K	L	S	K	K	<b>G</b>	E	K	V	<b>I</b>	<b>T</b>	<b>I</b>	K	-	345																			
Hv	349	-	E	G	Q	D	V	A	I	P	C	Q	V	T	G	L	P	E	P	K	V	S	-	368																			
Hv	1079	-	G	A	A	G	C	C	A	A	G	A	T	G	T	G	G	C	T	A	T	C	C	C	T	T	G	C	C	A	A	A	A	A	A	-	1138						
Hv	1072	-	G	A	A	G	C	C	A	A	G	A	T	G	T	G	G	C	T	A	T	C	C	C	T	T	G	C	C	A	A	A	A	A	A	-	1131						
Hv	346	-	E	G	Q	D	V	A	I	P	C	Q	V	T	G	L	P	E	P	K	V	S	-	365																			
qRT-PCR Hv Hem R																																											
Hv	369	-	<b>F</b>	T	Y	N	A	K	<b>P</b>	L	G	<b>E</b>	R	A	<b>I</b>	Y	K	D	G	V	L	T	-	388																			
Hv	1139	-	T	T	T	A	C	A	T	A	C	G	T	A	A	A	C	C	C	T	T	G	T	G	A	G	A	G	C	T	A	T	C	T	A	C	A	A	A	-	1198		
Hv	1132	-	T	G	G	A	C	A	T	A	A	G	G	T	C	T	T	G	T	G	A	C	A	G	A	G	C	T	A	C	T	T	A	A	A	A	-	1191					
Hv	366	-	<b>W</b>	T	Y	N	A	K	<b>G</b>	L	G	<b>D</b>	R	A	<b>T</b>	Y	K	D	G	V	L	T	-	385																			
Hv	389	-	I	K	N	A	K	K	G	D	T	G	Y	Y	G	C	K	A	E	N	E	H	-	408																			
Hv	1199	-	A	T	T	A	A	A	A	C	G	C	A	A	A	G	G	G	T	G	A	C	T	G	G	T	T	A	C	T	A	T	G	A	T	G	-	1258					
Hv	1192	-	A	T	C	A	A	A	C	G	T	A	A	A	G	G	T	G	A	C	T	G	G	A	C	T	G	G	A	A	T	G	A	A	T	G	-	1251					
Hv	386	-	I	K	N	A	K	K	G	D	T	G	Y	Y	G	C	K	A	E	N	E	H	-	405																			
Hv	409	-	G	D	L	Y	A	E	T	L	V	H	V	A	*								-	420																			
Hv	1259	-	G	G	T	A	T	T	G	T	A	G	T	G	T	G	A	C	C	T	T	G	T	G	T	T	A	G	T	T	A	G	T	T	A	G	-	1298					
Hv	1252	-	G	G	A	C	T	T	G	T	A	G	T	G	T	G	A	C	C	T	T	G	T	T	G	T	T	A	G	T	T	A	G	T	T	A	G	-	1311				
Hv	406	-	G	D	L	Y	A	E	T	L	V	H	V	A	*								-	417																			
Hv	1312	-	T	T	A	G	T	T	T	A	T	T	G	C	C	A	A	T	T	G	T	A	T	T	T	T	A	T	T	A	T	T	A	T	T	A	-	1371					
Hv	1372	-	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	-	1387					

Fig. 1. (Continued).

The minimum evolution tree was sought using Branch-and-Bound search with 2000 bootstrap replicates with *M. sexta* Neuroglian (AAC47451) as outgroup. The minimum evolution (ME) tree was searched using the Close-Neighbor-Interchange algorithm [31] at a search level of 1. The Nearest Neighbor-Joining algorithm [32] was used to generate the initial tree. All positions containing gaps and missing data were eliminated from the dataset.

### 3. Results

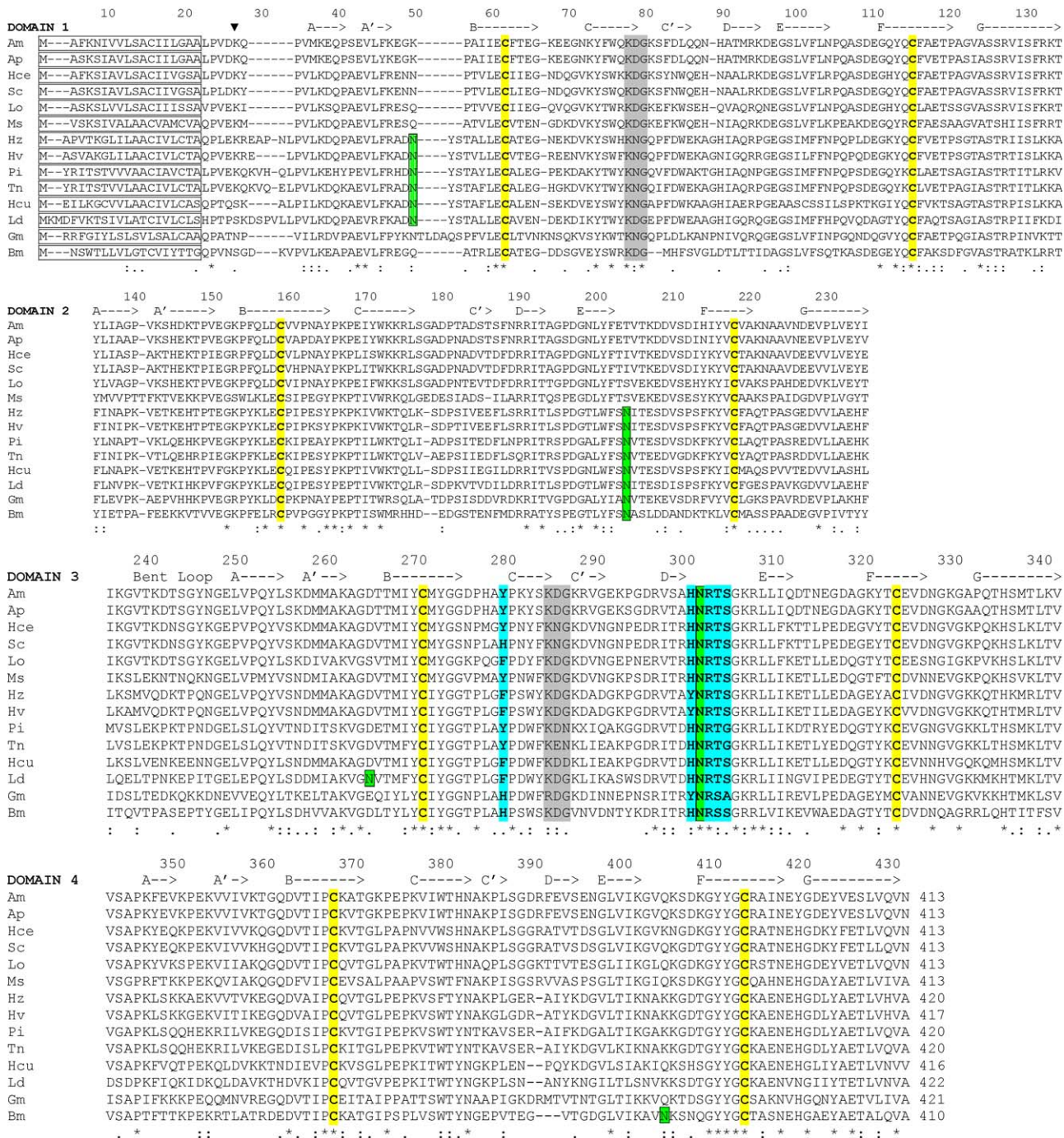
#### 3.1. Cloning of Hemolin cDNA

A 600 bp fragment (exon 4 according to the gene structure of Hemolin from *H. cecropia* [33]) was cloned from *H. virescens* pupal

DNA. This sequence was used to design primers for 5'- and 3'RACE reactions. The full-length *H. virescens* cDNA sequence was determined to be 1387 bp encoding 417 amino acids with a 19 residue predicted signal sequence (Fig. 1). The entire *H. zea* Hemolin cDNA was cloned using *H. virescens* full-length primers. In comparison with *H. virescens*, *H. zea* possessed three more amino acids (Fig. 1).

#### 3.2. Amino acid sequence analysis

Inclusion of amino acid sequences from several species presented in this paper make apparent that all species belonging to Noctuoidea have two extra N-glycosylation sites (positions 49 and 203, Fig. 2). The *H. zea* and *H. virescens* amino acid sequences



**Fig. 2.** Alignment of *Hemolin* amino acid sequences from 14 species of Lepidoptera. The sequences are ordered into structural domains and  $\beta$ -sheet locations deduced from the X-ray structure of Hemolin from *H. cecropia* (GenBank PDB no. 1BIH). The signal sequences are boxed. The eight conserved cysteine residues forming disulfide bridges are shown in bold with yellow background. KGD/KNG sites are marked in grey and N-glycosylation sites are marked in green the latter ones are also boxed. Amino acids predicted to be involved in phosphate binding are shown in bold with turquoise background. Am, *A. mylitta*; Ap, *A. pernyi*; Hce, *H. cecropia*; Sc, *S. cynthia*; Lo, *L. obliqua*; Ms, *M. sexta*; Hz, *H. zea*; Hv, *H. virescens*; Pi, *P. includens*; Tn, *T. ni*; Hcu, *H. cunea*; Ld, *L. dispar*; Gm, *G. mellonella*; Bm, *B. mori*. ▼ indicates start of predicted structures.

are most similar to the other species in the Noctuoidea superfamily (Fig. 3). Following the classification by Betts and Russell [34], only two of the 33 amino acids (8%) that differed between the two species are non-preferred substitutions (Fig. 1).

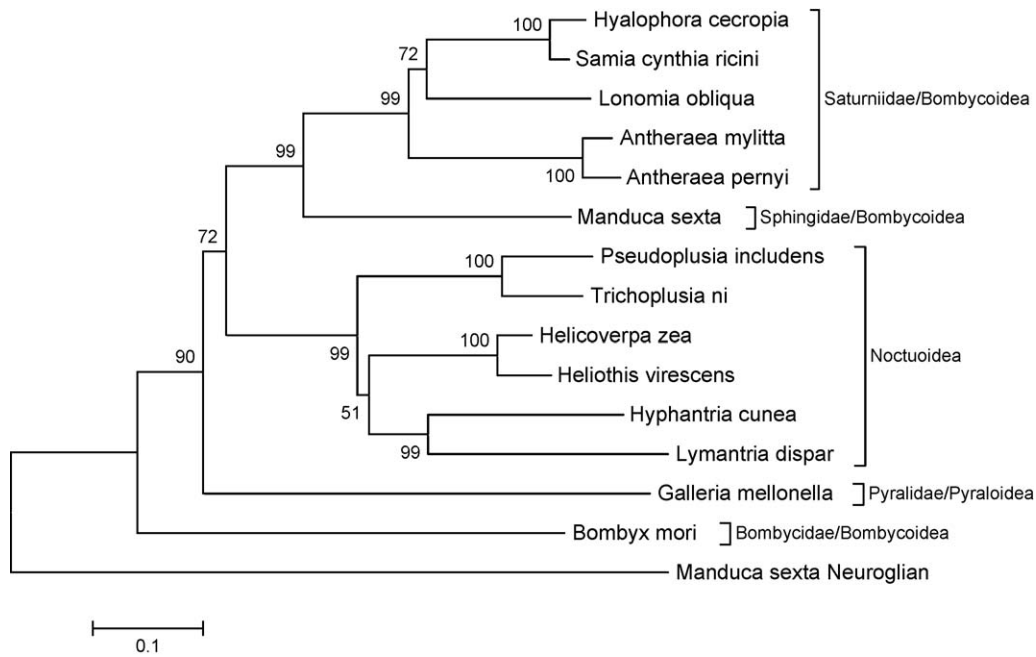
### 3.3. Phylogenetic analysis

The phylogenetic tree (Fig. 3) groups Noctuoidea together, but as in the previous tree based on *Hemolin* from 6 species [35], the current tree containing 14 species splits the superfamily Bombycoidea so

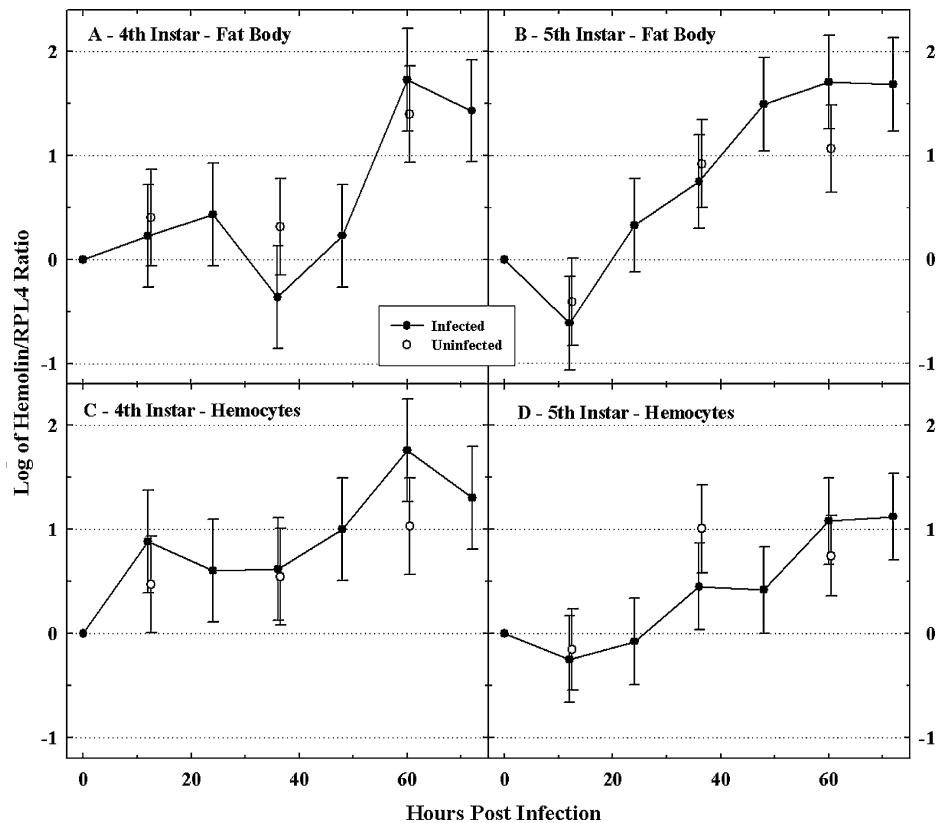
that the separation of Bombycidae from Saturniidae and Sphingidae is maintained. Thus, when it previously was an open question whether the *Hemolin* gene sequences were following the species phylogeny, it now seems as if they do, and that *Bombyx mori* is the oddity, being separated from the other macrolepidopterans.

### 3.4. Viral infection of larvae

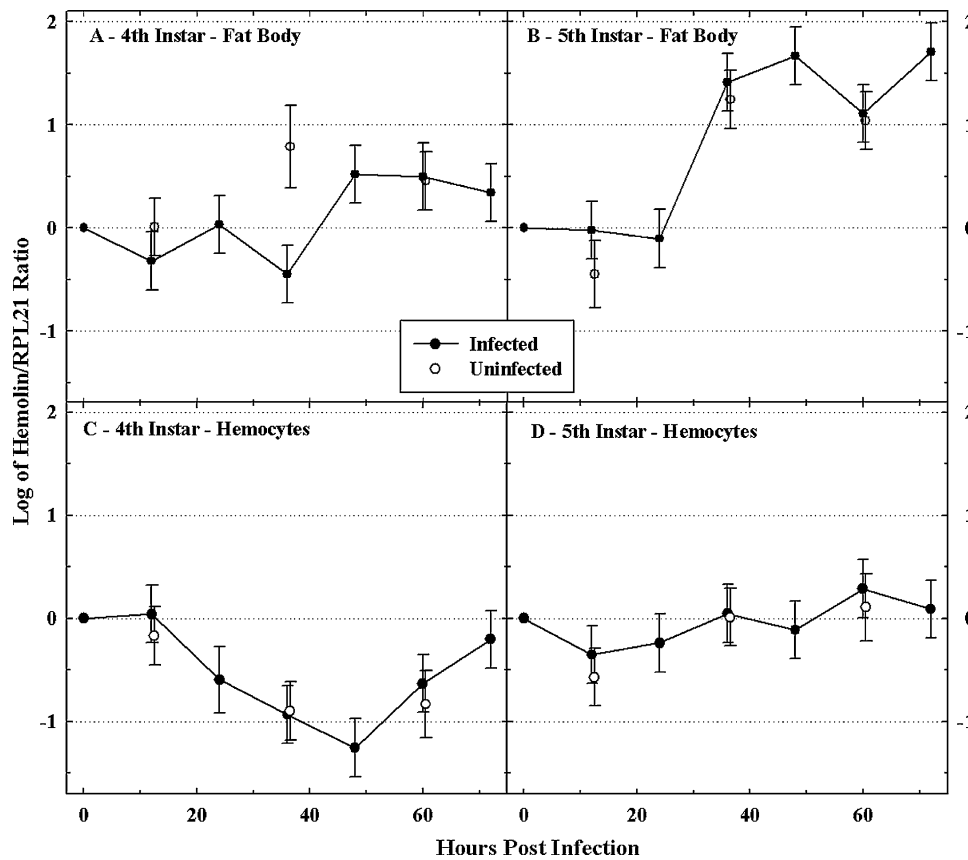
In initial assays, *H. virescens* and *H. zea* neonate larvae had similar LT<sub>50</sub>s (time of 50% larval death) when larvae were infected



**Fig. 3.** Phylogenetic tree of *Hemolin* amino acid sequences obtained after Branch-and-Bound search with the minimum evolution algorithm using *M. sexta Neuroglan* as outgroup (four most N-terminal domains). The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (2000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site.



**Fig. 4.** Quantitative PCR analysis of *Hemolin* expression in fat bodies and hemocytes of 4th and 5th instar larval *H. virescens* following infection with HzSNPV. Expression data were normalized to the expression of ribosomal protein L4 (*HvRPL4*). (Panel A) Expression in fat bodies dissected from 4th instar larvae. (Panel B) Expression in fat bodies dissected from 5th instar larvae. (Panel C) Expression in hemocytes collected from 4th instar larvae. (Panel D) Expression in hemocytes collected from 5th instar larvae. Filled symbols represent virus-infected tissues. Open symbols represent tissues from mock-infected larvae (LS mean  $\pm$  SE,  $n = 3$ ).



**Fig. 5.** Quantitative PCR analysis of *Hemolin* expression in fat bodies and hemocytes of 4th and 5th instar *H. zea* larvae following infection with HzSNPV. Expression data were normalized to the expression of ribosomal protein L4 (*HvRPL21*). (Panel A) Expression in fat bodies dissected from 4th instar larvae. (Panel B) Expression in fat bodies dissected from 5th instar larvae. (Panel C) Expression in hemocytes collected from 4th instar larvae. (Panel D) Expression in hemocytes collected from 5th instar larvae. Filled symbols represent tissues from virus-infected larvae. Open symbols represent tissues from mock-infected larvae (LS mean  $\pm$  SE,  $n = 3$ ).

with HzSNPV,  $75.4 \pm 3.7$  and  $72.7 \pm 1.4$  h post-infection (hpi), respectively (mean  $\pm$  SEM,  $n = 30$ ). Infected 4th instar larvae did molt into 5th instar larvae at approximately 36 h as did mock-infected larvae. However fatally infected larvae did not begin to burrow in diet in preparation for pupation, thereby differing from mock-infected larvae. By 72 hpi, a portion of the virus-infected larvae had died or had become blackened in appearance.

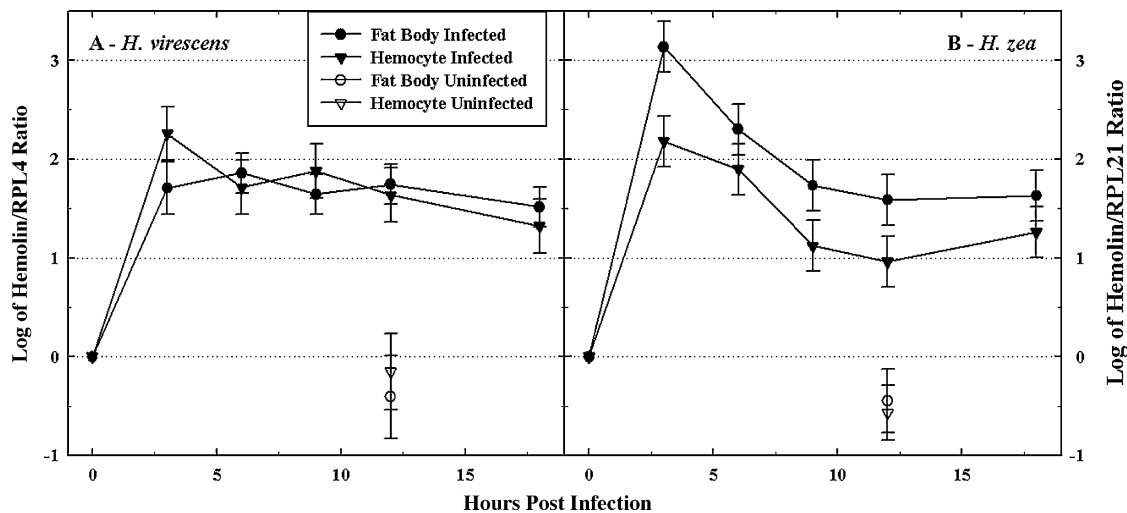
### 3.5. Expression analysis of *Hemolin*

Real-time quantitative (qPCR) was used to measure *Hemolin* expression during the course of microbial infection of *H. virescens* and *H. zea* larvae. Expression of *Hemolin* was measured in the hemocytes and fat bodies of 4th and 5th instar larvae of each species following *per os* infection with HzSNPV at 0, 12, 24, 36, 48, 60 and 72 hpi (Figs. 4 and 5). When larvae were infected *per os* at the early 4th instar stage they continued to develop and moulted to the 5th instar at 36 hpi before succumbing to infection 3 days post-infection. Larvae infected at the early 5th instar also continued to develop for 3 days post-infection, up to the time of death. Therefore, tissues from mock-infected larvae were included to assess normal developmental levels of *Hemolin* expression in the absence of baculoviral infection. *H. virescens Hemolin* transcript data were normalized to the expression of ribosomal protein L4 (*HvRPL4*) transcripts while *H. zea Hemolin* transcription were normalized to expression of *H. zea* ribosomal protein L21 (*HvRPL21*).

*H. virescens Hemolin* transcription in fat bodies dissected from larvae infected at the onset of the 4th instar was significantly elevated at 60 hpi and 72 hpi (Fig. 4A). Larvae infected during the

4th instar molted to the 5th instar at 36 h in well-synchronized larvae. The increase in *Hemolin* transcription is correlated with physiological changes in larvae occurring during the progression of the 5th instar. No significant differences were observed between tissues from HzSNPV-infected and mock-infected larvae (Fig. 4A). *Hemolin* transcription in 5th instar fat bodies increased throughout the 72 h period observed (Fig. 4B). In this experiment there also was no significant difference between infected and uninfected fat bodies (Fig. 4B). Hemocytic transcription of *Hemolin* trended upward during the 4th instar and 5th instar periods (Fig. 4C and D). A similar increase in *Hemolin* transcription also was noted in mock-infected individuals, which indicates that *H. virescens Hemolin* transcription was unaffected by baculoviral infection in both 4th and 5th instar larval fat bodies and hemocytes.

*H. zea Hemolin* transcription in 4th instar fat bodies changed little during the 72 h time-course of HzSNPV infection (Fig. 5A). A single significant difference between mock-infected controls and HzSNPV-infected tissues was evident at 36 hpi, which may coincide with the beginning of the moult to the 5th instar in this species. However, as this is the sole incidence of a significant difference between experimental and mock-infected controls, and because there are no reports in the literature of an HzSNPV related delay or disruption of larval/larval metamorphosis, we are reluctant to credit this divergence from the pattern observed in the other tissues and instars. *Hemolin* fat-body transcription following HzSNPV infection differed slightly between instars over the 72 h time course of infection (Fig. 5A and B). *Hemolin* transcription by the fat-body in 4th instar larvae remained low (Fig. 5A). By contrast, in 5th instar larval fat bodies *Hemolin* transcription did not change up until 24 h, after which



**Fig. 6.** Quantitative PCR analysis of *Hemolin* expression in fat bodies and hemocytes of 5th instar *H. virescens* and *H. zea* larvae following injection of *Micrococcus luteus*. (Panel A) Log transformed qPCR ratio of *H. virescens Hemolin* to *HvRPL4* during the course of infection. (Panel B) Log transformed qPCR ratio of *H. zea Hemolin* to *HvRPL21* following bacterial infection. Filled symbols represent tissues from bacteria-infected larvae. Open symbols represent tissues from mock-infected larvae (LS mean  $\pm$  SE,  $n = 3$ ).

transcription was significantly elevated for the remaining time observed. In 5th instar larvae there was no difference in transcription pattern between control mock-infected larvae, and HzSNPV-infected larvae (Fig. 5B).

*H. zea Hemolin* transcription patterns in 4th instar hemocytes exhibited a depressed rate from 12 hpi to 48 hpi, after which the transcript level increased again to control levels seen at the beginning of the infection period (Fig. 5C). *Hemolin* transcription was lowest early in the 5th instar. No significant differences were seen between mock-infected and HzSNPV-infected tissues (Fig. 5C). Hemocytic transcription of *Hemolin* did not appear to change significantly during the 5th instar period examined (Fig. 5D). As was seen in the fat bodies of 5th instar larvae, *Hemolin* transcription was not significantly different from mock controls (Fig. 5D). This indicates strongly that *Hemolin* transcription was unaffected by baculoviral infection in 5th instar larval fat bodies and hemocytes.

The lack of response to baculoviral infection prompted us to test the immuno-responsiveness of *Hemolin*. Thus as a control, the expression of *H. virescens Hemolin* and *H. zea Hemolin* in 5th instar larvae during the first 18 h (0, 3, 6, 9, 12 and 18 h) of a bacterial infection was studied (Fig. 6). When 5th instar *H. virescens* larvae were pricked with a tungsten needle dipped into a suspension of heat-killed Gram<sup>+</sup> and Gram<sup>-</sup> bacteria, a burst of *H. virescens Hemolin* transcription in hemocytes and fat bodies was evident, which peaked within 3 hpi (Fig. 6A). Elevated *H. virescens Hemolin* transcription was sustained throughout the entire 18 hpi, and there was no difference in the pattern of expression between the two tissues (Fig. 6A). Similarly, when 5th instar *H. zea* larvae were immunostimulated, transcription of *H. zea Hemolin* in both fat bodies and hemocytes rose rapidly to a peak at 3 hpi, then declined slightly before leveling off for the remaining 18 hpi (Fig. 6B). Throughout the monitoring period, transcription of *H. zea Hemolin* was significantly higher in fat bodies than in hemocytes (Fig. 6B).

#### 4. Discussion

*Hemolin* was originally identified as the most abundant protein expressed after bacterial injection into *H. cecropia* [10]. Recently, it was proposed that *Hemolin* also can be involved in viral defense [12]. However, although *Hemolin* seems to be present throughout Lepidoptera and is induced in response to bacterial infection in all species investigated, the involvement in viral infection is less

studied and could be an adaptation occurring in only some phylogenetic groups of Lepidoptera [36]. In this paper, we have performed the first study of *Hemolin* expression after natural baculovirus infection in noctuid moths and showed a lack of response to the infection. Our data corroborates the current literature, which suggests that *Hemolin* may be involved in viral infection in bombycid moths, but not in noctuid moths. Thus, baculovirus infection leading to up-regulation has been shown in *A. pernyi* (Saturniidae), but is also reported as unpublished observations in *H. cecropia* (Saturniidae) and *B. mori* (Bombycidae) [35]. In addition, a polydnal protein binds to *Hemolin* from *M. sexta* (Sphingidae) [14]. In contrast, in *Trichoplusia ni* (Noctuidae), feeding or injection of baculovirus did not induce *Hemolin* as indicated by the lack of binding by an *H. cecropia Hemolin* antibody [37]. Also, preliminary data show that in *T. ni* and *Pseudoplusia includens* (Noctuidae), *Hemolin* is induced by injection of *E. coli*, but not by AcMNPV (M.H. Beck, O. Terenius, M. Strand, unpublished). Likewise, in the noctuid moth *S. frugiperda* no *Hemolin* up-regulation was observed among 1750 ESTs expressed in response to polydnal virus infection [38].

In our experiments, the HzSNPV baculovirus infection did not change *Hemolin* expression levels in either *H. zea* or *H. virescens*, which is in line with the decreased expression of prophenolox- idases-1 and -2 observed in HzSNPV-infected 4th instar *H. virescens* larvae, and the constant level of PPO expression seen in HzSNPV infected 5th instar larvae [18]. Similarly, RNAi of *Hemolin* in bacteria-infected *H. cecropia* resulted in decreased prophenolox- idase levels [39] demonstrating the intimate connection between *Hemolin* and phenoloxidase. In contrast, prophenoloxidase subunit transcription was up-regulated by ichnoviral but not by densoviral infection of *S. frugiperda* larvae [38]; however, neither of these treatments significantly affected *Hemolin* transcription. As expected though, a strong up-regulation of *Hemolin* expression was seen in response to bacterial infection similar to the increased prophenoloxidase subunit transcript levels in response to bacterial infection observed in *H. zea* and *H. virescens* [18].

Although *Hemolin* transcript levels did not change in response to baculoviral infection, they changed in response to develop- mental stage. Larval *H. virescens* exhibit a broad peak of hemolymph 20-hydroxyecdysone (20E) during the 4th instar directly preceding the premolt stage; 20E then declines to low levels during the molt to the 5th instar [40]. Thus, when the early 4th instar *H. virescens* larvae were subject to increasing ecdysteroid

titers, fat body and hemocyte *Hemolin* transcription was accelerating. As larvae molt into the 5th instar, creating a milieu of increasing JH-III titer [40], fat body and hemocyte *Hemolin* transcription plateaued. As larvae progress through the 5th instar, the JH-III titer drops precipitously and the ecdysone titer increases to levels well above those of the late 4th instar. It is during this dramatic increase that *Hemolin* transcription levels increased in fat bodies as well as hemocytes. This argues for a direct effect of ecdysteroid concentration on *Hemolin* transcription in *H. virescens* larvae, similar to other moth species. Developmental expression of *Hemolin* has previously been found in *Lymantria dispar* [41], *M. sexta* [42] and more recently, also in the Indian meal moth *Plodia interpunctella* [43] and the Wax moth *Galleria melonella* [44] both belonging to Pyralidae (Pyraloidea), a moth family outside Macrolepidoptera. Both *L. dispar* and *P. interpunctella* larvae treated with the ecdysteroid biosynthesis inhibitor KK-42 experienced reduced levels of *Hemolin* mRNA confirming the ecdysteroid involvement [41,43]. Notably, in the up-stream regions of the *Hemolin* genes of *M. sexta* and *H. cecropia* there are putative ecdysone-responsive elements [45], which presumably are involved in the *Hemolin* regulation. Although no endocrine data are available for larval *H. zea*, we had assumed that the close relatedness of these two species would infer a high degree of mechanistic similarity. However, little change was seen in *Hemolin* transcription in 4th and 5th instar *H. zea* larvae except in the fat body of 5th instars where an increase in *Hemolin* transcription was seen in a similar timing to that of 5th instar fat bodies from *H. virescens*.

Since a long larval period leads to prolonged baculovirus multiplication, it is an advantage for the baculovirus to decrease the impact of the molting hormone ecdysone and slow down the molting process. Therefore, ecdysone levels are reduced during baculovirus infection in LdMNPV of *L. dispar* and AcMNPV of *S. frugiperda* by the baculovirus-produced ecdysteroid UPD-glucosyl-transferase (*egt*), which is an enzyme that conjugates ecdysteroids with galactose or glucose [46–48]. During an infection of *H. zea* larvae with HzSNPV, deletion of the *egt* gene from the viral genome did not impact the speed of fatal infection [49], consistent with the interpretation that larvae may be able to compensate for the conjugation of ecdysone by increasing the production of ecdysone.

The difference in viral response between bombycoid and noctuid moths is intriguing. If *Hemolin* is up-regulated in response to depletion of free *Hemolin* being bound to microorganisms, the binding properties of *Hemolin* may be a factor determining the up-regulation. Perhaps the lack of response after virus injection in *H. zea* and *H. virescens* could be due to a weaker binding. Notably, all noctuid moths in the alignment presented in this paper have a phenylalanine in position 279, which earlier has been suggested to bring a weaker binding property to the phosphate binding site HNRTS in domain 3 [35]. Alternatively, the suggested virus-response elements present in the up-stream regions of *Hemolin* from *H. cecropia* and *M. sexta* [45] might not be present in noctuid moths.

In summary, our results indicate that although *Hemolin* transcription is strongly induced by bacterial infection; its expression following a baculovirus infection differs between noctuid moths and silk moths. These results suggest that order-specific immunological adaptations, such as the presence of *Hemolin* only in the Lepidoptera, could on a functional level even extend to family-specific adaptations.

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